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Mechanical and material property changes in bone with experimental diabetes: a murine model

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by

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Mechanical and material property changes in bone with experimental diabetes: a murine model

Thesis directed by Professor Virginia L. Ferguson

Over 23 million people in the United States are plagued by diabetes mellitus. Studies have shown that those with diabetes have a higher occurrence of bone fractures than those without the disease, but risk factors associated with diabetes account for only a portion of these fractures (Schwartz 2003; Vestergaard 2006). This implies that there are properties of diabetic bones that account for this increase in fractures.

Research has been performed on the bone mineral density (BMD) of those with and without diabetes. However, studying this singular property of bones does not fully explain the increase in bone fractures in the diabetic population. Additionally, this research has yielded inconclusive results: some studies show that diabetes leads to an increase in BMD while others show a decrease in this property (Krakauer et al. 1995; Retzepi & Donos 2010).

The objective of this study is to analyze the effect of experimental diabetes on bone properties in mice. This relationship is explored through analyzing different mouse strains to determine a potential genetic link, kidney removal to mimic the effects of nephropathy, and an LXR agonist to explore a potential diabetes treatment. We hypothesize that diabetes will negatively impact bone properties across multiple strains of mice, kidney removal will further degrade bone properties and the LXR agonist will improve some of the negative bone effects that diabetes causes.

In general, STZ-induced diabetes was accompanied by decreases in physical and compositional bone properties in both strains of mice. Kidney removal and treatment with an LXR-agonist had little to no effects on these bone properties. Kidney removal was performed in skeletally mature mice; this old age may have contributed to the lack of effects. Similarly, the LXR agonist was administered over a short time period, and the detrimental effects from the diabetes were not able to be overcome. The data presented here provides evidence that experimentally-induced diabetes corresponds with a decrease in bone properties and nephropathy. While LXR agonists hold promise for mitigating bone property changes in diabetes, further analysis is required to determine their potential effects.

Contents

1. Background

1.1 Diabetes Mellitus and Liver X Receptors

Diabetes mellitus is a group of metabolic diseases characterized by chronic glucose concentrations higher than those which the kidneys are capable of reabsorbing, resulting from one's inability to correctly produce or use insulin. Of primary importance in blood glucose control are the pancreatic β-cells, which produce, store and release insulin into the blood stream at appropriate times. In healthy individuals, these β-cells are continuously creating and storing this insulin within the cell. Insulin is then released into the blood stream in small amounts throughout the day and night, ensuring that the body has a continual supply of blood glucose even when time has passed since a meal. As this insulin level drops, the liver is signaled to add more glucose to the blood stream by converting stored glycogen into glucose and releasing it. In addition to this continuous monitoring, the β-cells immediately release their stored insulin when a meal is consumed. These β-cells then monitor blood glucose concentration levels and appropriately secrete more insulin into the blood stream. After this first insulin release, the β-cells pause and then again monitor the blood glucose levels. If they are too high, another insulin response occurs, returning the blood glucose level to its original pre-meal level. This combination of two insulin responses immediately after meals and a continual monitoring of blood glucose levels between meals ensures that healthy individuals maintain appropriately low blood glucose levels. When these insulin responses do not occur in this fashion, blood glucose levels rise and do not return to normal, leading to a diabetic state.

Those with type 1 diabetes mellitus (T1DM) do not produce insulin, leading to an excess amount of glucose in the blood stream. This diabetic phenotype is due to damage or loss of pancreatic β-cells, the cells responsible for producing insulin. Without insulin, the appropriate

insulin responses to blood glucose levels cannot occur, and these levels rise. By contrast, those with type 2 diabetes mellitus (T2DM) either do not produce enough insulin or are insulin-resistant. The muscle and liver cells require higher than normal levels of insulin to store glucose, causing large amounts of glucose to remain in the blood. Additionally, this insulin deficiency is then erroneously interpreted by the liver cells as a sign that blood glucose levels are too low, when indeed they are too high. These cells then release more glucose into the blood stream, further raising blood glucose levels.

The liver X receptors α and β are regulators of, among other functions, glucose homeostasis. They are activated by LXR agonists. Once activated, they dimerize and recruit additional proteins required for transcription. Due to their relationship with glucose homeostasis, LXR agonists are beginning to be researched as potential anti-diabetic drugs (Cao 2002; Efanov et al. 2004; Kase et al. 2007). LXR agonists have been found to decrease plasma glucose levels and improve insulin sensitivity in rats, demonstrating their viability as a diabetic treatment (Cao 2002). Furthermore, LXR agonists have been shown to reduce inflammatory activity of bone marrow-derived macrophages, demonstrating that these agonists may improve bone properties (Kiss et al. 2011; Lakomy et al. 2009).

1.2 Kidney Disease

One potential negative effect of diabetes is kidney disease. In healthy individuals the kidneys filter waste out of the blood. However, diabetes damages blood vessels in the kidneys which diminishes the ability of the kidneys to properly filter the blood and eventually causes kidney disease. Because the kidneys are responsible for regulating levels of calcium and phosphorus in the blood, those with chronic kidney disease may have poor bone properties (Morrone et al. 2011; Sherrard et al. 1993; Stompór et al. 2011).

1.3 Murine Model

Murine models have been frequently used to analyze various properties of bone. Mice are easy to maintain, can be easily induced with diabetes, and yield information on bone that can be correlated to human bone. Additionally, there are a variety of strains of mice, each with their own specific properties. In one of the studies implemented here, two different mouse strains were compared to determine how diabetes affects bones with inherently different properties.

Two mouse strains used frequently for bone analyses are C57Bl/6J and DBA/2J. As compared to another common mouse strain, C3H/HeJ, both C57Bl/6J and DBA/2J mice have a lower BMD. In one study analyzing 12-month old female mice, or mice at peak bone maturity, the C57Bl/6J mice were found to have a nearly 50% lower femoral BMD and the DBA/2J mice were found to have a 17% lower femoral BMD than the C3H/HeJ mice (Beamer et al. 1996). Similar differences have been seen in 4-month old female mice, with C57Bl/6J mice having an 18% lower BMD and DBA/2J mice having a 17% lower BMD than the C3H/HeJ mice (M. P. Akhter et al. 2000). Morphological measurements also differ across mouse strains. At the femoral mid-diaphysis, the total cross-sectional area is larger in C57Bl/6J mice than DBA/2J mice while the cortical thickness is larger in the DBA/2J mice than the C57Bl/6J mice (M. P. Akhter et al. 2000; Beamer et al. 1996). Therefore the mid-diaphyseal moment of inertia is smaller in DBA/2J mice than C57Bl/6J mice (M. P. Akhter et al. 2000). Body weights have been found to be the same across the two mouse strains, but DBA/2J mice have been found to have shorter femora than C57Bl/6J (M. P. Akhter et al. 2000). Biomechanical femoral strength results, namely ultimate and yield stresses have been found to be lower in C57Bl/6J mice as compared to DBA/2J mice, rendering them weaker (M. P. Akhter et al. 2000). A table displaying the relevant differences between the two strains is shown below in Table 1.

Table 1: Differences in bone properties among two mouse strains.

Bone Property Differences Between Mouse Strains			
Property	**Comparison**	**C57Bl/6J**	**DBA/2J**
BMD	Qualitative Comparison	Low	Low
	Femoral BMD (mg/cm^2)[i]	57.83 ± 0.84	58.25 ± 1.77
Moment of Inertia	Qualitative Comparison	Large	Small
	Femur (mm^4)[i]	0.106 ± 0.011	0.058 ± 0.017
Cortical Thickness	Qualitative Comparison	Small	Large
	Femoral Ct.Th (mm)[ii]	0.292	0.379

Citations are marked as: [i] (M. P. Akhter et al. 2000), [ii] (Beamer et al. 1996).

In addition to these differing bone properties, the diabetic phenotype differs with mouse strain. Both C57Bl/6J and DBA/2J mice can develop diabetes from streptozotocin (STZ) (T. A Einhorn et al. 1988; Erdal et al. 2010; Kanter et al. 2007), yet DBA/2J mice tend to show a more significant diabetic phenotype as compared to the C57Bl/6J mouse (Breyer et al. 2005; Brosius et al. 2009).

1.4 Drug-induced Diabetes Using Streptozotocin

T1DM can be easily induced in the two chosen mouse strains, C57Bl/6J and DBA/2J, using STZ (T. A Einhorn et al. 1988; Erdal et al. 2010; Kanter et al. 2007). STZ has been successfully used to induce diabetes for decades (Rossini et al. 1977). When used in a murine model it is injected intravenously for multiple consecutive days and creates a diabetic state by killing the insulin-producing (and thus glucose-regulating) pancreatic β-cells. Because STZ is structurally similar to glucose, it is transported into the β-cells by the glucose transporter GLUT2. It then damages DNA through alkylation, leading to a diabetic state (Szkudelski 2001). In addition to increased blood glucose levels, STZ causes a decrease in bone volume, mineral apposition rate, and osteocalcin serum and tibia messenger RNA levels (Motyl & McCabe 2009). It also causes an increase in bone marrow adiposity and aP2 expression (Motyl & McCabe

2009). STZ-induced diabetic mice exhibit a bone phenotype consistent with human studies and spontaneously diabetic mouse models, confirming that it is an acceptable model for T1DM bone loss (Motyl & McCabe 2009).

2. Motivation

2.1 Diabetes and Bone

Research has been performed on the bone mineral density of those with diabetes and those without the disease. However, studying this singular property of bones does not fully explain the increase in bone fractures in the diabetic population as compared to the normal population. Additionally, this research on bone mineral density has yielded inconclusive results: some studies show that diabetes can lead to an increase in bone mineral density while others show that it can lead to a decrease in this property (Krakauer et al. 1995; Retzepi & Donos 2010; Vestergaard 2006). This lack of understanding of the changes in properties of bone with diabetes leaves a large gap in the research that remains unexplained. Analysis of concurrent material and mechanical property changes of diabetic and healthy bone is needed.

2.2 Diabetes' Effects on Murine Bone

Only a few studies have been performed that attempt to quantify the changes in mouse and rat bone with diabetes (Dixit & Ekstrom 1980; T. A Einhorn et al. 1988; Erdal et al. 2010; Kanter et al. 2007; Prisby et al. 2008; Verhaeghe et al. 1994; L. Zhang et al. 2008). These previous analyses focused on relatively limited scope of bone properties. The most marked differences are demonstrated by measurements of bone mineral density: some studies report a decrease in BMD (Prisby et al. 2008; L. Zhang et al. 2008) and others show no significant change in BMD (Erdal et al. 2010; Verhaeghe et al. 1994) with both experimentally-induced and endogenous diabetes. This difference in BMD is consistent with the literature analyzing human bone and diabetes and gives further evidence that the singular property of BMD cannot be a reliable method of determining bone strength with diabetes. The diabetic bone phenotype is likely influenced by extrinsic factors such as diet, environmental influences, and genetics.

In addition to bone mineral density, the literature yields some information on the mechanical properties of diabetic mouse and rat bone. Studies have consistently demonstrated that diabetes decreases the maximum force that long bones can withstand in bending tests (Dixit & Ekstrom 1980; T. A Einhorn et al. 1988; Erdal et al. 2010; Kanter et al. 2007; Prisby et al. 2008; G. K Reddy et al. 2001; Verhaeghe et al. 1994; L. Zhang et al. 2008). However, some studies show a compensatory increase in stiffness (T. A. Einhorn et al. 1988; G. K Reddy et al. 2001) while others show no change in this value (Prisby et al. 2008; Verhaeghe et al. 1994). This increased stiffness and decreased strength can lead to embrittlement and thus an increase in fracture risk. This may be caused by an increase in collagen cross-linking, as this cross-linking has been shown to increase matrix stiffness (Goh & Cooper 2008; G Kesava Reddy 2004).

The trabecular structure of mouse bone has also been studied using micro-CT. Two separate studies demonstrated that STZ-induced T1DM has a negative effect on bone. The relative bone volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) all decreased as compared to control values (Kanter et al. 2007; L. Zhang et al. 2008).

Although the literature shows some trends toward diabetes having a negative effect on mouse bone, a complete study analyzing multiple properties of bone across different mouse strains has not been performed. This thesis explores the link between genetics, diabetes, and bone properties using a mouse model.

3. Testing of Bone

3.1 Biomechanical Testing of Bone

The physical properties of bone can be determined through a variety of testing methods. Some of the most common methods used when analyzing a mouse model include three-point bending (J. Bonadio et al. 1990; Engesaeter et al. 1978; Lu et al. 2008; Matthew J Silva et al. 2005; Torzilli et al. 1981), four-point bending (M P Akhter et al. 1998; J Bonadio et al. 1993; C Kesavan & S Mohan 2010; Chandrasekhar Kesavan et al. 2005; Lewis et al. 1993), vertebral compression (M. P. Akhter et al. 2004; Court et al. 2007; Reeves et al. 2007), torsion (J. Bonadio et al. 1990; Ekeland et al. 1981; T. S. Keller et al. 1986; Lewis et al. 1993), and femoral neck fracture (Jämsä et al. 1998; Peng et al. 1994; Søgaard et al. 1994; Turner et al. 1996). Of these tests, bending tests (including three-point bending and femoral neck fracture) are some of the most simple to perform and are highly repeatable in mouse models (Jämsä et al. 1998; M. D Brodt et al. 1999). Additionally, they yield results which are equally useful yet have less measurement error as those from some of the more complex test methods, such as torsion testing (M. D Brodt et al. 1999). For these reasons, three-point bending and femoral neck fracture were chosen as the main mechanical testing methods for the studies presented herein.

During three-point bending, structural stiffness is affected by Young's modulus (E) and moment of inertia (I) and structural strength is affected by yield and ultimate stress ($\sigma_{y,u}$). E and $\sigma_{y,u}$ depend on the bone's composition, while I depends on the shape of the bone. I can be calculated and combined with the experimentally determined values of force and deflection to determine the meaningful structural properties of Young's modulus (E), yield stress (σ_y), and ultimate stress (σ_u).

3.2 Micro-CT

Micro-computed tomography (micro-CT) is a method used to determine the mechanical properties of bone by quantifying the trabecular bone in a given sample. Due to its complex anisotropic structure, quantifying trabecular bone is much more difficult than quantifying dense, cortical bone. Micro-CT creates a three-dimensional array of the bone nondestructively and yields quantitative values describing the bone, as shown below in Table 2. Micro-CT has been used to study bone for over a decade and is a fairly simple, nondestructive, and precise technique to describe the trabecular bone (Feldkamp et al. 1989; Rüegsegger et al. 1996). Increases in relative bone volume, connectivity density, trabecular number, and trabecular thickness and a decrease in trabecular separation represent positive changes in the bone (Hildebrand et al. 1999; Ma et al. 2011).

Table 2: Definitions of micro-CT indices.

Index	Abbreviation	Description	Positive Result
Total volume (mm^3)	TV	Volume of the entire region of interest	-
Bone volume (mm^3)	BV	Volume of the region segmented as bone	--
Relative bone volume (%)	BV/TV	Ratiof of the segmented bone volume to the total volume	Increase
Connectivity density (mm^{-3})	Conn.D	Degree to which structure is multiply connected	Increase
Trabecular number (mm^{-1})	Tb.N	Measure of the average number of trabeculae per unit length	Increase
Trabecular thickness (mm)	Tb.Th	Mean thickness of trabeculae	Increase
Trabecular spacing (mm)	Tb.Sp	Mean distance between trabeculae	Decrease

Table modified from (Bouxsein et al. 2010).

In one previously performed study, micro-CT was used to analyze new bone formation on untreated T1DM, insulin-treated T1DM, and healthy mouse bones. T1DM mice had lower BV/TV ($P<0.01$) and Tb.Th ($P<0.01$) tibiae values than healthy mice (Thrailkill et al. 2005). In another study, micro-CT was used to analyze bone formation during fracture healing in a T1DM model. Sixteen days after bone fracture, BV/TV was 38% higher in healthy mice than in the T1DM group ($P<0.05$) (Kayal et al. 2007). Another study showed DBA/2J mice with T1DM

had a lower BV/TV due to a decrease in Tb.Th and Tb.N at the femur metaphysic, yet Conn.D remained unchanged between the two groups. The decrease occurred by 10 weeks with diabetes and did not increase with age (Nyman et al. 2010). These changes in BV/TV and Tb.Th with T1DM in multiple studies show that T1DM may decrease trabecular bone properties.

3.3 Compositional Analysis

Ashing bones yields information on the mineral content quickly and easily. The compositional analysis performed in this study used ashing at two different temperatures to first determine the dry mass and then determine the mineral mass. The percent of bone that is mineralized can then be easily calculated from these two values:

$$\%Min = \frac{Min_M}{Dry_M} x100\%$$

Studies analyzing both bone mineral content and mechanical properties of bones demonstrate that a decrease in mineral content is accompanied by a decrease in mechanical properties, primarily yield load, peak load, and stiffness (Gordon et al. 1992; Ward et al. 2003). Additionally, diabetes has been shown to cause an increase in the mineral content of the diaphysis, or primarily cortical region, of diabetic bones as compared to healthy bones (T. A Einhorn et al. 1988).

3.4 Statistical Analysis

A complete statistical analysis was performed for all of the data from both of the studies. The first study analyzed the effect of diabetes and a potential treatment on two strains of mice and thus a three-way analysis of variance (ANOVA) should be performed. However, due to low n-values a three-way ANOVA would not yield meaningful results due to low statistical power. Therefore each strain was analyzed separately using a two-way ANOVA with follow-on t-tests using α=0.05. The second study analyzed the effect of diabetes and kidney disease on one strain

of mice, and thus a two-way ANOVA with follow on t-tests will be performed using $\alpha=0.05$. All data are presented as mean ± standard deviation.

4. Study 1: The Effect of Mouse Strain, Diabetes and Treatment on Bone Properties

4.1 Introduction

The objective of this study is to determine the differences of bone properties across two different strains of mice, both with and without STZ-induced T1DM and with and without a potential treatment, the LXR agonist GW3965. Multiple facets of the bone were studied: mechanical testing and compositional analysis were implemented to study the biomechanical properties and compositional properties, respectively.

To date there has not been a comprehensive study analyzing the effects of diabetes on bone across multiple mouse strains, yet the genetic differences in bone properties (M. P. Akhter et al. 2000; Beamer et al. 1996) and diabetic phenotype (Breyer et al. 2005; Brosius et al. 2009) imply a potential connection. Because the bone properties of these two mouse strains have been well-characterized (M. P. Akhter et al. 2000; Beamer et al. 1996), analyzing their differences with T1DM may yield valuable information on the effects of the disease on bones with differing properties.

We hypothesize that the diabetic bones will have decreased mechanical and compositional properties. More specifically, we expect the biomechanical testing, both at the primarily cortical mid-diaphysis and at the primarily trabecular femoral neck, to show decreased maximum load, stiffness, force at yield, and energy at yield with T1DM. We also expect the compositional analysis to show a decrease in the mineral content and mineral percentage of bone. The LXR agonist is hypothesized to improve these properties in diabetic mice and prevent detrimental property changes that are observed in diabetes.

4.2 Materials and Methods

This study aims to analyze differences between two mouse strains, both with and without diabetes (induced using STZ) and with and without the potential treatment, the LXR agonist GW3965. A total of n = 42 male, five and a half month old mice were used, as shown below in Table **3**.

Table 3: Study design.

	DBA/2J		**C57Bl/6J**	
	Control	STZ (Diabetes)	Control	STZ (Diabetes)
Placebo	n = 6	n = 6	n = 6	n = 3
LXR Agonist	n = 6	n = 3	n = 6	n = 6

To induce T1DM, 8-week old mice were injected with STZ (Sigma-Aldrich; St Louis, MO) intraperitoneally (40 mg/kg made freshly in 50mM sodium citrate buffer, pH 4.5) or with citrate solution only for five consecutive days. One week after the last STZ injection, tail vein blood glucose levels were measured to confirm the diabetic state where a blood glucose level of greater than 250 mg/dL was considered diabetic. Mice were fed a standard Western diet (Harlan-Teklad; Madison, WI). Once diabetes was confirmed, mice were randomized to receive either this standard Western diet only, for placebo mice, or this diet mixed with GW3965, for LXR-agonist treated mice (Rizzo et al. 2010).

Following all treatments, the mice were sacrificed at approximately five and a half months of age and frozen at -80 °C. Mice were then thawed and the femora, humeri, and tibiae were removed and cleaned of all non-osseous tissue. Cleaned femora and humeri were wrapped in phosphate-buffered saline dampened gauze and frozen at -80 °C. Cleaned tibiae were stored in ethanol at room temperature.

4.2.1 Physical Measurements

Final body masses of each mouse were measured immediately prior to sacrifice and lean body masses of each mouse were measured during dissection. Femora and humeri lengths were measured with calipers and wet masses of these bones were measured using a digital scale.

4.2.2 Mechanical Testing

The right femora were measured for length and wet mass and were defrosted at room temperature for two hours prior to mechanical testing in three-point bending and femoral neck bending (Insight 2, MTS Systems Corporation, Eden Prairie, MN). All samples were tested to failure at a deflection rate of 5 mm/min. The bones were tested in three-point bending using a custom anvil with an 8 mm span. Each bone was placed in the identical position on the anvil prior to testing. To test the strengths of the femoral necks, the femora were held in a vice parallel to the femoral shaft axis. Each femur was oriented such that the neck and head were pointed upward, out of the vice. The load was applied directly to the femoral head until failure occurred.

All mechanical tests yielded force-deflection curves which were analyzed for stiffness, force and energy at the elastic limit, maximum force and energy, and force and energy at fracture, as shown below in Figure 1.

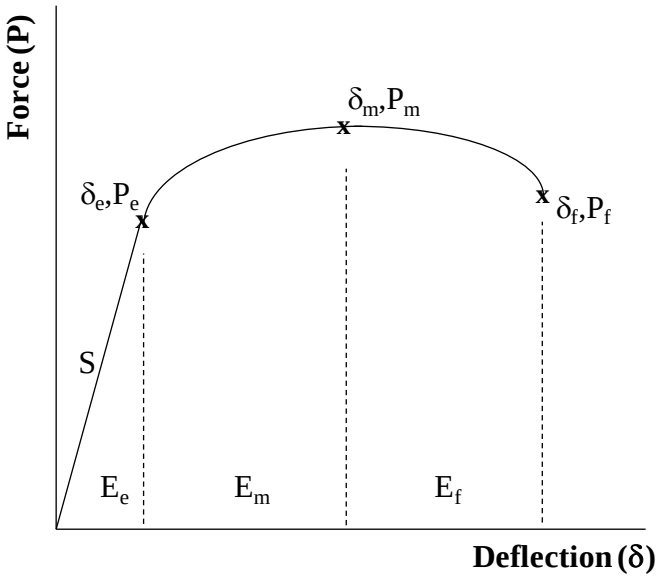

Figure 1: Force vs. deflection curve (Ferguson 2001).

These structural properties were combined with the moment of inertia of the bones to determine the material properties of Young's Modulus (E), yield stress (σ_y), and ultimate stress (σ_u) as shown:

$$I_x = \frac{\pi}{64}\left(D_{oML}{}^3 D_{oAP} - D_{iML}{}^3 D_{iAP}\right)$$

$$I_y = \frac{\pi}{64}\left(D_{oAP}{}^3 D_{oML} - D_{iAP}{}^3 D_{iML}\right)$$

$$z = \frac{D_{oAP}}{2}$$

$$E = \frac{SL^3}{48 I_x}$$

$$\sigma = \frac{PLz}{4 I_x}$$

4.2.3 Compositional Analysis

Immediately following mechanical testing, all pieces of the broken bones (right femora and humeri) were oven-dried at 105 °C for 24 hours and then at 800 °C for 24 hours to obtain the

bone dry mass (Dry_M) and the bone mineral mass (Min_M), respectively. Percent mineralization (%Min) was calculated from these two values:

$$\%Min = \frac{Min_M}{Dry_M} x100\%$$

4.2.4 Embedding and Imaging

The left humeri were placed in ethanol for 24 hours and air-dried for 48 hours prior to embedding. A non-infiltrating epoxy (Epo-Quick, Buehler, Lake Bluff, IL) was used to embed the humeri. The bones were then sectioned using a low-speed saw at the mid-diaphysis, immediately distal to the trochanter.

The bones were then ground flat with a 340 grit paper and wheel polished up to a 9 µm diamond polish (Buehler Ltd.; Lake Bluff, IL). The humeri cross-sections were visualized (SpotCamera; Sterling Heights, MI) and digital images were captured at a 10x magnification (Zeiss Axioskop FL40; Thornwood, NY). Measurements of bone morphology were completed (ImageJ, National Institute of Health, Bethesda, Maryland) and included outer (periosteal) area (T.Ar), perimeter (Ps.Pm), and major- and minor-diameter (D_{oML} and D_{oAP}). They also included inner (medullary cavity) area (Ec.Ar), perimeter (Ec.Pm), and major- and minor-diameter (D_{iML} and D_{iAP}). Cortical area (Ct.Ar) and thickness (Ct.Th) were calculated:

$$Ct.Ar = T.Ar - Ec.Ar$$

$$Ct.Th = \frac{Ct.Ar}{\frac{Ps.Pm + Ec.Pm}{2}}$$

4.3 Results

4.3.1 Physical Measurements

Physical measurements of body mass, lean body mass, tibia length and humerus length were taken for all C57Bl/6J and DBA/2J mice (Table 4.) Both strains of mice showed decreased

body mass and lean body mass with diabetes yet exhibited no significant differences in bone length. In the C57Bl/6J mice the body mass was 19.2% larger in the control mice than in the diabetic mice (P=0.005) and the lean body mass was 34.4% larger in the control mice than in the diabetic mice (P<0.001). Similarly, in the DBA/2J mice the body mass was 67.4% larger in the control mice than in the diabetic mice (P<0.001) and the lean body mass was 53.1% larger in the control mice than in the diabetic mice (P<0.001).

Table 4: Measured body mass, lean body mass, tibia length and humerus length for C57Bl/6J and DBA/2J mice.

C57Bl/6J					
Variable	Control/Placebo (n=6)	Diabetes/Placebo (n=3)	Control/LXR Agonist (n=6)	Diabetes/LXR Agonist (n=6)	Two-way ANOVA
Body Mass (g)	27.0 ± 3.2	24.3 ± 0.2	28.9 ± 2.3[a]	22.4 ± 4.1[a]	Con >> STZ
Lean Body Mass (g)	9.6 ± 0.5[a, b]	7.6 ± 0.4[a]	11.1 ± 0.5b[a, b]	7.2 ± 1.6[a]	Con >>> STZ
Tibia Length (mm)	18.02 ± 0.26	17.74 ± 0.19	18.44 ± 0.86	17.89 ± 0.17	--
Humerus Length (mm)	12.42 ± 0.28	12.16 ± 0.04	12.59 ± 0.16	12.21 ± 0.17	--

DBA/2J					
Variable	Control/Placebo (n=6)	Diabetes/Placebo (n=6)	Control/LXR Agonist (n=6)	Diabetes/LXR Agonist (n=3)	Two-way ANOVA
Body Mass (g)	42.9 ± 10.6[a]	20.0 ± 1.3[a]	40.5 ± 1.6[a]	22.1 ± 4.7[a]	Con >>> STZ
Lean Body Mass (g)	13.9 ± 0.8[a, b]	7.6 ± 0.6[a, b]	11.0 ± 0.6[a, b]	6.4 ± 1.5[a, b]	Con >>> STZ
Tibia Length (mm)	17.6 ± 0.29	17.24 ± 0.51	17.61 ± 0.27	17.46 ± 0.22	--
Humerus Length (mm)	11.97 ± 0.17	11.64 ± 0.22	11.98 ± 0.14	11.77 ± 0.18	--

Data are presented as mean ± standard deviation. Two-way ANOVA differences are presented in the far right column to show the pooled results between the diabetic and non-diabetic groups and the LXR agonist-treated and untreated groups. Two-way ANOVA differences are marked as Con>>>STZ if $P \leq 0.001$, Con>>STZ if $0.001 < P < 0.01$ and Con>STZ if $0.01 \leq P < 0.05$. Statistically significant differences between groups from post-hoc student t-tests are shown as [a]non-diabetic vs. diabetic for either placebo-treated or LXR agonist-treated groups, [b]placebo-treated vs. LXR agonist-treated for either non-diabetic or diabetic groups and [c]differences between C57Bl/6J and DBA strains for either non-diabetic or diabetic groups.

4.3.2 Mechanical Testing

The three-point bending tests yielded information about the humeri at the mid-diaphysis. The measured forces and calculated stresses are shown below in

Table 5

In the C57Bl/6J mice the maximum load in the control groups was 20.7% larger than in the diabetic groups (P=0.017), the force at yield for the control groups was 31.9% larger than in the diabetic groups (P=0.007), and the energy to yield for the control groups was 53.5% larger than in the diabetic groups (P=0.019).

In the DBA/2J mice the maximum load in the control groups was 17.1% larger than in the diabetic groups (P=0.016), the stiffness was 30.1% larger than in the diabetic groups (P<0.001), and the force at yield in the control groups was 23.12% larger than in the diabetic groups (P=0.007).

Table 5: Measured and calculated mechanical testing data for C57Bl/6J and DBA/2J right humeri, both with and without diabetes and without and without the LXR agonist treatment. Bones were tested in three-point bending at the mid-diaphysis.

C57Bl/6J					
Variable	Control/Placebo (n=6)	Diabetes/Placebo (n=3)	Control/LXR Agonist (n=6)	Diabetes/LXR Agonist (n=6)	Two-way ANOVA
S (N/mm)	66.3 ± 12.8	60.0 ± 12.3	62.5 ± 15.0	55.8 ± 5.1	--
P_e (N)	9.1 ± 1.4	7.6 ± 0.9	10.0 ± 2.3[a]	6.6 ± 1.7[a]	Con >> STZ
P_m (N)	11.2 ± 1.5	10.1 ± 1.1	12.8 ± 1.2[a, c]	10.2 ± 1.9[a]	Con > STZ
E_e (mJ)	1.0 ± 0.4	0.7 ± 0.1	1.1 ± 0.6[a]	0.6 ± 0.1[a]	Con > STZ
σ_e (N/mm^2)	108.9 ± 22.0[c]	97.4 ± 21.5	112.0 ± 35.8	91.3 ± 18.7	--
σ_m (N/mm^2)	133.0 ± 15.7[c]	129.8 ± 26.7	140.6 ± 20.5	132.0 ± 23.0	--

DBA/2J					
Variable	Control/Placebo (n=5)	Diabetes/Placebo (n=6)	Control/LXR Agonist (n=6)	Diabetes/LXR Agonist (n=3)	Two-way ANOVA
S (N/mm)	64.1 ± 6.6[a]	47.7 ± 8.6[a]	66.5 ± 8.8[a]	49.5 ± 9.1[a]	Con >>> STZ
P_e (N)	9.5 ± 1.3	7.6 ± 2.0	9.7 ± 1.7[a]	6.2 ± 2.1[a]	Con >> STZ
P_m(N)	11.3 ± 1.8	9.6 ± 1.6	11.2 ± 0.9[c]	9.2 ± 1.1	Con > STZ
E_e (mJ)	0.9 ± 0.3	0.7 ± 0.2	0.8 ± 0.5	0.5 ± 0.4	--
σ_e (N/mm^2)	153.8 ± 38.4[c]	129.9 ± 41.3	114.0 ± 32.1	102.4 ± 41.6	--
σ_m (N/mm^2)	181.6 ± 41.7[c]	151.3 ± 46.9	155.7 ± 39.1	148.3 ± 29.4	--

Data are presented as mean ± standard deviation. Two-way ANOVA differences are presented in the far right column to show the pooled results between the diabetic and non-diabetic groups

and the LXR agonist-treated and untreated groups. Two-way ANOVA differences are marked as Con>>>STZ if P≤0.001, Con>>STZ if 0.001<P<0.01 and Con>STZ if 0.01≤P<0.05. Statistically significant differences between groups from post-hoc student t-tests are shown as [a]non-diabetic vs. diabetic for either placebo-treated or LXR agonist-treated groups, [b]placebo-treated vs. LXR agonist-treated for either non-diabetic or diabetic groups and [c]differences between C57Bl/6J and DBA strains for either non-diabetic or diabetic groups.

Three-point bending tests were also performed on the right femora of the diabetic and non-diabetic groups of only the DBA/2J strain. The measured forces are shown below in Table 6. The stiffness in the control groups was 37.6% larger than in the diabetic groups (P=0.002), the force at yield for the control groups was 43.1% larger than in the diabetic groups (P=0.004), the maximum force for the control groups was 28.7% larger than in the diabetic groups (P=0.003), and the energy to yield for the control groups was 43.6% larger than in the diabetic groups (P=0.040).

Table 6: Measured mechanical testing data for DBA/2J femora, both with and without diabetes. Bones were tested in three-point bending at the mid-diaphysis

DBA/2J			
Variable	Control/Placebo (n=6)	Diabetes/Placebo (n=6)	Student t-Test
S (N/mm)	105.6 ± 10.1	72.2 ± 17.2	Con >> STZ
P_e (N)	16.5 ± 3.1	10.7 ± 2.3	Con >> STZ
P_m(N)	21.4 ± 2.3	16.0 ± 2.5	Con >> STZ
E_e (mJ)	1.5 ± 0.5	1.0 ± 0.3	Con > STZ
E_m (mJ)	3.5 ± 1.1	2.4 ± 0.6	--

Data are presented as mean ± standard deviation. Differences from t-tests are presented in the far right column to show the significant differences between the diabetic and non-diabetic groups. t-Test differences are marked as Con>>>STZ if P<0.001, Con>>STZ if 0.001<P<0.01 and Con>STZ if 0.01<P<0.05.

Additionally, femoral-neck bending tests were performed on the right femora of the diabetic and non-diabetic groups of only the DBA/2J strain. The maximum load in the control

groups was 65.7% larger than in the diabetic groups (P<0.001) and the energy to maximum for the control groups was 73.2% larger than in the diabetic groups (P=0.002).

DBA/2J			
Variable	Control/Placebo (n=6)	Diabetes/Placebo (n=6)	Student t-Test
S (N/mm)	73.1 ± 25.0	63.4 ± 20.1	--
P_e (N)	6.3 ± 2.2	6.2 ± 1.9	--
P_m(N)	21.4 ± 3.0	10.8 ± 2.4	Con >>> STZ
E_e (mJ)	0.5 ± 0.2	0.5 ± 0.3	--
E_m (mJ)	6.1 ± 1.3	2.8 ± 1.4	Con >> STZ

Data are presented as mean ± standard deviation. Differences from t-tests are presented in the far right column to show the significant differences between the diabetic and non-diabetic groups. t-Test differences are marked as Con>>>STZ if P<0.001, Con>>STZ if 0.001<P<0.01 and Con>STZ if 0.01<P<0.05.

4.3.3 Compositional Analysis

The measured length, wet mass, dry mass, mineral mass and calculated percent mineral content for each of the right humeri are shown below in Table 7.

In both mouse strains the mineral mass and percent mineral content of the bones was significantly larger in the control groups. In the C57Bl/6J mice the mineral mass in the diabetic groups was 23.2% smaller than in the control groups (P<0.001) and the percent mineral content in the diabetic groups was 3.16% smaller than in the control groups (P=0.028), yet no significant differences in bone length, wet mass, or dry mass were observed. Similar differences occurred in the composition of the DBA/2J mouse bones where the mineral mass in the control groups was 27.7% larger than in the diabetic groups (P<0.001) and the percent mineral content in the control groups was 3.34% larger than in the diabetic groups (P=0.003). The DBA/2J mice also showed no significant differences in bone length, wet mass, or dry mass.

Table 7: Composition of C57Bl/6J and DBA/2J right humeri as a function of experimental diabetes and LXR agonist treatment.

C57Bl/6J					
Variable	Control/Placebo (n=6)	Diabetes/Placebo (n=3)	Control/LXR Agonist (n=6)	Diabetes/LXR Agonist (n=6)	Two-way ANOVA
Length (mm)	12.42 ± 0.28	12.16 ± 0.04	12.59 ± 0.16	12.21 ± 0.17	--
Wet Mass (mg)	29.7 ± 2.1	27.9 ± 1.1	33.0 ± 1.8	27.5 ± 2.5	--
Dry Mass (mg)	19.8 ± 1.1	17.2 ± 0.3	20.8 ± 1.3	16.3 ± 2.0	--
Mineral Mass (mg)	12.1 ± 0.9[a]	10.4 ± 0.4[a]	13.2 ± 0.8[a]	9.8 ± 1.5[a]	Con >>> STZ
Percent Mineral (%)	60.8 ± 2.1 [b]	60.3 ± 1.4[a]	63.4 ± 0.5[b]	60.1 ± 2.2[a]	Con > STZ

DBA/2J					
Variable	Control/Placebo (n=6)	Diabetes/Placebo (n=6)	Control/LXR Agonist (n=6)	Diabetes/LXR Agonist (n=3)	Two-way ANOVA
Length (mm)	11.97 ± 0.17	11.64 ± 0.22	11.98 ± 0.14	11.77 ± 0.18	--
Wet Mass (mg)	28.2 ± 1.7	22.9 ± 1.5	26.9 ± 1.6	22.5 ± 3.3	--
Dry Mass (mg)	19.2 ± 1.4	14.5 ± 0.6	18.6 ± 1.3	15.4 ± 2.1	--
Mineral Mass (mg)	12.2 ± 1.0[a]	9.1 ± 0.5[a]	12.1 ± 1.0[a]	9.7 ± 1.4[a]	Con >>> STZ
Percent Mineral (%)	65.2 ± 1.3[a]	62.9 ± 1.3[a]	65.2 ± 1.5	63.3 ± 0.4	Con >> STZ

Data are presented as mean ± standard deviation. Two-way ANOVA differences are presented in the far right column to show the pooled results between the diabetic and non-diabetic groups and the LXR agonist-treated and untreated groups. Two-way ANOVA differences are marked as Con>>>STZ if $P \leq 0.001$, Con>>STZ if $0.001 < P < 0.01$ and Con>STZ if $0.01 \leq P < 0.05$. Statistically significant differences between groups from post-hoc student t-tests are shown as [a]non-diabetic vs. diabetic for either placebo-treated or LXR agonist-treated groups, [b]placebo-treated vs. LXR agonist-treated for either non-diabetic or diabetic groups and [c]differences between C57Bl/6J and DBA strains for either non-diabetic or diabetic groups.

4.3.4 Imaging and Cross-Section Measurements

Images for each of the humeri were taken and representative images for each strain are shown below in Figure 2.

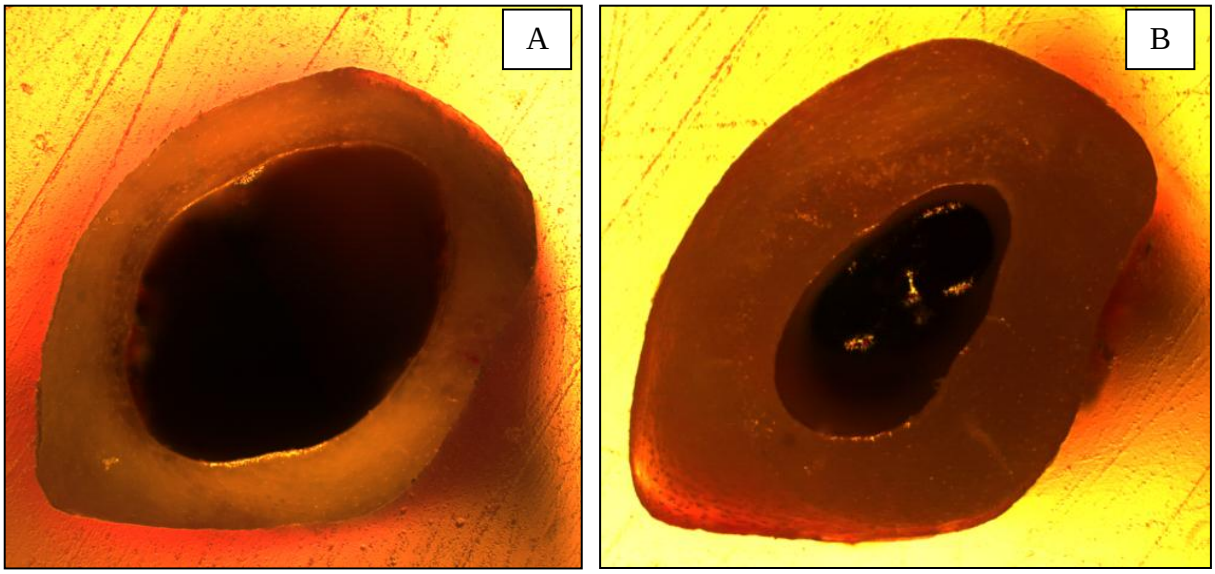

Figure 2: Humeri cross-section from (A) C57Bl/6J mouse and (B) DBA/2J mouse. Field width of images is 1.51mm.

As shown in Figure 2 and as was expected from previous research (M. P. Akhter et al. 2000; Beamer et al. 1996), the cortical thickness of the C57Bl/6J mouse is smaller than that of the DBA mouse. All measured and calculated bone morphology values are shown below in Table 8. In both strains of mice the cortical thickness was significantly larger in the control mice than in the diabetic mice. The C57Bl/6J mice had an 18.9% larger thickness ($P<0.001$) and the DBA/2J mice had a 9.9% larger thickness ($P=0.041$).

Table 8: Experimental and calculated morphological measurements of left humerus of C57Bl/6J and DBA/2J mice, with and without diabetes and with and without an LXR agonist treatment.

C57Bl/6J					
Variable	Control/Placebo (n=6)	Diabetes/Placebo (n=3)	Control/LXR Agonist (n=6)	Diabetes/LXR Agonist (n=6)	Two-way ANOVA
D_{iML} (mm)	0.81 ± 0.04	0.82 ± 0.03[b]	0.82 ± 0.03[a]	0.89 ± 0.05[a, b]	STZ > Con
D_{iAP} (mm)	0.58 ± 0.03	0.60 ± 0.02[b]	0.63 ± 0.07	0.67 ± 0.04[b]	--
D_{oML} (mm)	1.27 ± 0.05	1.25 ± 0.03	1.30 ± 0.05	1.25 ± 0.05	--
D_{oAP} (mm)	0.92 ± 0.04[b]	0.93 ± 0.02	0.978 ± 0.07[b]	0.94 ± 0.03	--
Ps.Pm (mm)	3.41 ± 0.10	3.36 ± 0.07	3.53 ± 0.14[a]	3.39 ± 0.07[a]	--
Ec.Pm (mm)	2.20 ± 0.06	2.23 ± 0.02[b]	2.28 ± 0.14[a]	2.46 ± 0.11[a, b]	STZ > Con
Ec.Ar (mm^2)	0.36 ± 0.02	0.37 ± 0.00[b]	0.39 ± 0.05[a]	0.45 ± 0.03[a, b]	--
T.Ar (mm^2)	0.85 ± 0.05[b]	0.84 ± 0.03	0.92 ± 0.08[a, b]	0.85 ± 0.03[a]	--
Ct.Ar (mm^2)	0.49 ± 0.04	0.47 ± 0.03[b]	0.52 ± 0.04[a]	0.40 ± 0.05[a, b]	Con >> STZ
Ct.Th (mm)	0.18 ± 0.01	0.17 ± 0.01[b]	0.18 ± 0.01[a]	0.14 ± 0.02[a, b]	Con >>> STZ

DBA/2J					
Variable	Control/Placebo (n=6)	Diabetes/Placebo (n=6)	Control/LXR Agonist (n=6)	Diabetes/LXR Agonist (n=3)	Two-way ANOVA
D_{iML} (mm)	0.53 ± 0.03	0.58 ± 0.07	0.54 ± 0.03	0.58 ± 0.08	
D_{iAP} (mm)	0.32 ± 0.03	0.35 ± 0.04	0.32 ± 0.04	0.36 ± 0.06	STZ > Con
D_{oML} (mm)	1.13 ± 0.08	1.09 ± 0.10	1.16 ± 0.08	1.12 ± 0.09	
D_{oAP} (mm)	0.82 ± 0.02	0.82 ± 0.04	0.82 ± 0.02	0.83 ± 0.02	
Ps.Pm (mm)	3.04 ± 0.13	2.96 ± 0.20	3.01 ± 0.2	3.02 ± 0.13	
Ec.Pm (mm)	1.34 ± 0.08	1.47 ± 0.17	1.37 ± 0.10	1.49 ± 0.20	
Ec.Ar (mm^2)	0.13 ± 0.02	0.15 ± 0.03	0.13 ± 0.02	0.16 ± 0.04	STZ > Con
T.Ar (mm^2)	0.68 ± 0.04	0.66 ± 0.08	0.69 ± 0.05	0.67 ± 0.03	
Ct.Ar (mm^2)	0.55 ± 0.04	0.50 ± 0.08	0.56 ± 0.08	0.52 ± 0.07	
Ct.Th (mm)	0.25 ± 0.02	0.23 ± 0.03	0.25 ± 0.02	0.23 ± 0.04	Con > STZ

Data are presented as mean ± standard deviation. Two-way ANOVA differences are presented in the far right column to show the pooled results between the diabetic and non-diabetic groups and the LXR agonist-treated and untreated groups. Two-way ANOVA differences are marked as Con>>>STZ if $P \leq 0.001$, Con>>STZ if $0.001 < P < 0.01$ and Con>STZ if $0.01 \leq P < 0.05$. Statistically significant differences between groups from post-hoc student t-tests are shown as [a]non-diabetic vs. diabetic for either placebo-treated or LXR agonist-treated groups, [b]placebo-treated vs. LXR agonist-treated for either non-diabetic or diabetic groups and [c]differences between C57Bl/6J and DBA strains for either non-diabetic or diabetic groups.

4.4 Summary of Results

The objective of this study was to determine the mechanical and compositional changes in long-bones from male C57Bl/6J and DBA/2J mice, both with and without STZ-induced T1DM and with and without the LXR agonist GW3965, used as a potential treatment for T1DM. It was expected for the mechanical and compositional properties to decrease with T1DM in comparison to healthy bones, and for the LXR agonist-treated mice to have properties equivalent to the healthy mice.

Both strains of mice showed similar changes with T1DM and with the LXR agonist. The mechanical testing demonstrated significant decreases in the mechanical properties of the diabetic bones as compared to the non-diabetic bones across both strains. The maximum load and load at the elastic limit found from three-point bending at the primarily cortical mid-diaphysis were significantly decreased for both strains with T1DM, showing a decrease in their mechanical properties as compared to non-diabetic bones. The femoral neck testing showed that the maximum load of the DBA/2J diabetic bones was significantly lower than that of the non-diabetic bones, showing that there were decreases in this primarily trabecular region, as well.

Compositional analysis showed a decrease in the mineral content and the percent mineralization with T1DM in both mouse strains. These values decreased without a decrease in wet mass or length. These decreases in mineral content and percent mineralization demonstrate inferior compositional bone properties with T1DM.

The LXR agonist, GW3965, appeared to have no effect on the properties of bone: no increases in any properties of bone were seen with the agonist. The mineral losses the bones experienced appeared to be outweighed by any positive effects the agonist could have had.

5. Study 2: Changes in Bone Properties with Diabetes and Kidney Removal

5.1 Introduction

This second study aimed to determine the effects of diabetes and kidney removal on the biomechanical and material properties of bone in a single strain of mice: C57Bl/6J. Mechanical testing in three-point bending and femoral neck bending were used to determine the mechanical properties of bone and micro-CT tests were used to determine the material properties of bone.

Similarly to the first study, we hypothesize that the diabetic bones will have decreased mechanical and material properties as compared to the non-diabetic bones. We also hypothesize that the mice with only one kidney will have decreased bone mechanical and material properties as compared to the bones of mice with two kidneys. More specifically, we expect the biomechanical testing, both at the primarily cortical mid-diaphysis and at the primarily trabecular femoral neck, to show decreased maximum load, stiffness, force at yield and energy at yield with both diabetes and kidney removal. Additionally, we expect the micro-CT tests to show negative results, or a decrease in BV/TV, Conn.D, Tb.N, and Tb.Th and an increase in Tb.Sep with diabetes and kidney removal.

5.2 Materials and Methods

A total of n = 20 C57Bl/6 mice were studied, n = 5 mice per group with the two factors being tests of diabetes or nephropathy, with levels of diabetic and non-diabetic and two kidneys or one kidney (Table 9).

Table 9: Study design.

	Diabetic	Non-Diabetic
Two Kidneys	n = 5	n = 5
One Kidney	n = 5	n = 5

Kidney removal was performed at approximately three months of age. At five months of age diabetes was induced in the same manner as in the first study. Following all treatments, the mice were sacrificed at approximately one year of age; thus the mice can be considered skeletally mature (V. L Ferguson et al. 2003). Mice were frozen at -80 °C, thawed, and the femora and tibiae were removed and cleaned of all non-osseous tissue. Cleaned femora were wrapped in phosphate-buffered saline dampened gauze and frozen at -80 °C and cleaned tibiae were stored in ethanol at room temperature.

5.2.1 Physical Measurements

Femora and tibiae lengths were measured with calipers and wet masses of these bones were measured using a digital scale.

5.2.2 Mechanical Testing

The right femora were mechanically tested using the same procedure as was implemented in the first study. They were tested both at the mid-diaphysis and at the femoral head. As was performed in the first study, these tests yielded force-deflection curves which were analyzed for stiffness, force and energy at the elastic limit, maximum force and energy, and force and energy at fracture.

5.2.3 Micro-CT

Micro-CT tests were conducted on the left tibiae. An x-ray was used to illuminate the bone then a detector was used to collect the magnified images. These images were then compiled to create a complete image of the bone from which various quantitative values for trabecular bone were gathered.

5.3 Results

5.3.1Physical Measurements

Physical measurements of femora length and wet mass and tibiae length and wet mass were taken for all left side bones (Table 10). As indicated, there were no significant differences seen for lengths or masses for either femora or tibiae.

Table 10: Physical measurements of right femora and tibiae.

Variable	Two Kidney, Non-Diabetic (n=5)	One Kidney, Non-Diabetic (n=5)	Two Kidney, Diabetic (n=4)	One Kidney, Diabetic (n=5)	Two-way ANOVA
Femur Length (mm)	15.57 ± 0.14	15.37 ± 0.89	15.28 ± 0.88	15.58 ± 0.62	--
Tibiae Length (mm)	18.29 ± 0.24	18.42 ± 0.37	18.34 ± 0.27	18.01 ± 0.34	--
Femur Wet Mass (mg)	70.7 ± 6.0	69.5 ± 10.0	60.3 ± 6.4	64.5 ± 4.1	--
Tibiae Wet Mass (mg)	56.6 ± 5.7	56.3 ± 5.5	51.2 ± 4.0	50.3 ± 4.4	--

5.3.2 Mechanical Testing

Three-point bending and femoral neck bending were performed on the left femora of each of the mice. The three-point bending results are shown below in Table 11. Although there were no significant differences between the diabetic and non-diabetic groups, there were trends toward the diabetic groups having less favorable properties than the non-diabetic groups, including lower stiffness values and lower maximum load values. The stiffness of the groups with one kidney was found to be 38.8% larger than the groups with two kidneys (P=0.035).

Table 11: Measured and calculated mechanical testing data for C57Bl/6J right femora, both with and without diabetes and with two kidneys and one kidney. Bones were tested in three-point bending at the mid-diaphysis.

Variable	Two Kidney, Non-Diabetic (n=5)	One Kidney, Non-Diabetic (n=5)	Two Kidney, Diabetic (n=4)	One Kidney, Diabetic (n=5)	Two-way ANOVA
S (N/mm)	72.3 ± 12.3	91.5 ± 24.8	38.0 ± 25.6	77.5 ± 26.1	1 Kid > 2 Kid
P_e (N)	10.9 ± 3.5	10.7 ± 1.4	8.3 ± 2.6	9.4 ± 2.9	--
P_m (N)	14.9 ± 2.1[a]	15.3 ± 0.9	10.1 ± 3.0[a]	13.9 ± 4.6	--
E_e (mJ)	1.3 ± 1.1	1.1 ± 0.5	1.2 ± 0.4	1.0 ± 0.5	--

Data are presented as mean ± standard deviation. Two-way ANOVA differences are presented in the far right column to show the pooled results between the diabetic and non-diabetic groups and the two kidney and one kidney groups. Two-way ANOVA differences are marked as 1 Kid >>>2 Kid if $P \leq 0.001$, Con>>STZ if $P < 0.01$ and Con>STZ if $0.01 \leq P < 0.05$.
Statistically significant differences between groups from post-hoc student t-tests are shown as [a]non-diabetic vs. diabetic for either two kidney or one kidney groups, [b]two kidney vs. one kidney for either non-diabetic or diabetic groups.

Femoral neck tests yield information about the properties of this area of the bone and are shown below in

Table 12. The force at yield for the control groups was 46.1% larger than in the diabetic groups (P=0.005) and the maximum force for the control groups was 30.3% larger than in the diabetic groups (P=0.014). Additionally, the energy of the elastic limit for the two kidney groups was 52.5% larger than in the one kidney groups (P=0.049).

Table 12: Measured and calculated mechanical testing data for C57Bl/6J right femoral necks, both with and without diabetes and with two kidneys and one kidney.

Variable	Two Kidney, Non-Diabetic (n=5)	One Kidney, Non-Diabetic (n=5)	Two Kidney, Diabetic (n=4)	One Kidney, Diabetic (n=4)	Two-way ANOVA
S (N/mm)	73.5 ± 29.7	70.8 ± 28.1	38.5 ± 37.1	78.5 ± 55.8	--
P_e (N)	18.1 ± 1.6	15.9 ± 5.1[a]	12.3 ± 5.5	8.9 ± 2.5[a]	Con >> STZ
P_m (N)	18.9 ± 1.7	18.7 ± 2.9[a]	14.6 ± 4.4	11.1 ± 5.4[a]	Con > STZ
E_e (mJ)	2.8 ± 0.7	2.8 ± 1.7[a]	3.5 ± 2.2[b]	0.7 ± 0.4[a,b]	2 Kid > 1 Kid

Data are presented as mean ± standard deviation. Two-way ANOVA differences are presented in the far right column to show the pooled results between the diabetic and non-diabetic groups and the two kidney and one kidney groups. Two-way ANOVA differences are marked as 1 Kid >>>2 Kid if $P \leq 0.001$, Con>>STZ if $P < 0.01$ and Con>STZ if $0.01 \leq P < 0.05$.
Statistically significant differences between groups from post-hoc student t-tests are shown as [a]non-diabetic vs. diabetic for either two kidney or one kidney groups, [b]two kidney vs. one kidney for either non-diabetic or diabetic groups.

5.3.3 Micro-CT

The results of the micro-CT tests are shown below in Table 13. The trabecular thickness of the control groups was 18.0% larger than the diabetic groups ($P<0.001$) and the bone volume fraction of the control groups was 20.9% larger than the diabetic groups ($P=0.035$). Although there were no significant differences between the trabecular number, trabecular separation or connectivity density there were trends demonstrating that the diabetic groups had less favorable values for these properties.

Table 13: The results of the micro-CT tests.

Variable	Two Kidney, Non-Diabetic (n=5)	One Kidney, Non-Diabetic (n=5)	Two Kidney, Diabetic (n=5)	One Kidney, Diabetic (n=5)	Two-way ANOVA
Tb.N (1/mm)	3.33 ± 0.35	3.40 ± 0.33	3.34 ± 0.26	3.39 ± 0.09	--
Tb.Th (mm)	0.04 ± 0.01[a]	0.04 ± 0.004	0.03 ± 0.002[a, b]	0.04 ± 0.00[b]	Con >>> STZ
Tb.Sp (mm)	0.30 ± 0.03	0.29 ± 0.03	0.30 ± 0.02	0.29 ± 0.01	--
Conn.D. ($1/mm^3$)	33.6 ± 22.4	41.2 ± 25.7	37.2 ± 8.5	36.6 ± 10.1	--
BV/TV (%)	0.07 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	Con > STZ

Data are presented as mean ± standard deviation. Two-way ANOVA differences are presented in the far right column to show the pooled results between the diabetic and non-diabetic groups and the two kidney and one kidney groups. Two-way ANOVA differences are marked as 1 Kid >>>2 Kid if $P \leq 0.001$, Con>>STZ if $P < 0.01$ and Con>STZ if $0.01 \leq P < 0.05$.
Statistically significant differences between groups from post-hoc student t-tests are shown as [a]non-diabetic vs. diabetic for either two kidney or one kidney groups, [b]two kidney vs. one kidney for either non-diabetic or diabetic groups.

5.4 Summary of Results

Due to small group sizes few significant differences were seen. However, the mechanical testing data showed a trend toward a decrease in stiffness, force at yield, maximum force and energy at yield with T1DM at the femoral mid-diaphysis, a primarily cortical region of bone. The femoral neck testing data showed significant decreases in the force at yield and the maximum force with T1DM, indicating that there were decreased mechanical properties with T1DM in this primarily trabecular region of bone. Additionally, the micro-CT results showed a decrease in Tb.Th and BV/TV with T1DM, indicating that the diabetic bones were materially inferior to the non-diabetic bones. These results indicate that experimentally-induced diabetes could negatively impact the mechanical and material properties of bone in older mice.

Kidney removal appeared to have little effect on bone, potentially resulting from the skeletally-mature age of the mice. However, the micro-CT results showed a trend toward an

increase in trabecular bone volume with kidney removal, as expected from previous studies. Inducing kidney disease with a drug instead of simply removing one kidney would be expected to yield more pronounced results (Wilson 2008).

6. Discussion and Conclusions

The main objective of these studies was to quantify changes in bone properties with experimental diabetes. This link was explored through analyzing different mouse strains to determine a potential genetic link, kidney removal to mimic the effects of kidney disease, and an LXR agonist to explore a potential treatment for diabetes. In general, STZ-induced diabetes was accompanied by decreases in physical and compositional bone properties in both strains of mice and kidney removal and treatment with an LXR-agonist had little to no effects on these bone properties.

The mice with STZ-induced diabetes possessed less favorable compositional and biomechanical bone properties. Diabetes was accompanied by a decrease in mineral content and cortical thickness of the long bones, which likely caused the decreased biomechanical properties of the bone. These decreases in mineral and biomechanical properties demonstrate that diabetes has a negative effect on bone.

In general, there were more pronounced differences in the DBA/2J strain than the C57Bl/6J strain with diabetes, the LXR agonist treatment, and kidney removal. Although both of these mouse strains have been shown to be successfully induced with diabetes using STZ (T. A Einhorn et al. 1988; Erdal et al. 2010; Kanter et al. 2007), neither strain perfectly mimics the human disease (Breyer et al. 2005; Brosius et al. 2009). However, the more pronounced differences in bone properties in the DBA/2J strain provide evidence that the bone properties of this mouse strain may be more strongly affected by STZ, kidney removal, and the LXR agonist. Future analyses of diabetes with nephropathy and/or potential diabetic treatments can utilize this mouse strain to yield pronounced bone differences.

The LXR agonist GW3965 showed little to no differences in bone properties in either mouse strain analyzed. However, this LXR agonist has been shown to improve skeletal muscle cells and improve insulin secretion (Faulds et al. 2010) and was thus expected to improve the physical bone properties in this study. In this study, the LXR agonist was administered in skeletally mature mice for a short time period. Any changes in bone properties due to the LXR agonist may have been too minimal to improve the mineral losses the mice developed from their diabetic state. A more severe LXR agonist treatment, such as knocking out the LXR gene from the beginning of life may yield more pronounced results.

Kidney removal was associated with very little changes in bone properties. The diabetic state of the mice was associated with decreases in compositional and physical properties, and these negative effects overshadowed any further negative effects that kidney removal may have caused. Additionally, kidney removal on its own was not correlated with statistically significant decreases in bone properties, potentially resulting from the skeletally mature age of the mice. Although kidney removal can be used to mimic the effects of kidney disease, medically inducing nephropathy using a drug such as daunomycin may result in more pronounced effects (Wilson 2008). Medically inducing nephropathy at a skeletally immature age may also help resolve the lack of changes seen in the older mice studied here.

Overall, despite small group sizes STZ-induced diabetes caused many significant differences and trends in decreased mechanical, material, and compositional properties of bone across multiple strains. Although statistically significant bone changes from kidney removal or an LXR-agonist were not seen here, further studies with more severe methods of nephropathy and LXR knockout may result in more pronounced results.

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